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(54) Title: BONE AND CARTILAGE INDUCTIVE COMPOSITIONS

(57) Abstract

Purified BMP-3 proteins and processes for producing them are disclosed. Compositions thereof may be used in the treatment of bone and/or cartilage defects and in wound healing and related tissue repair.

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BONE AND CARTILAGE INDUCTIVE COMPOSITIONS

The present invention relates to a novel family of purified proteins designated BMP-3 proteins and processes for obtaining them. Compositions thereof may be used to induce bone and/or cartilage formation and in wound healing and tissue repair.

The invention provides proteins, capable of stimulating, promoting or otherwise inducing cartilage and/or bone formation, substantially free from other mammalian proteins. Human BMP-3 proteins of the invention are characterized by containing the amino acid sequence set forth in Table II from at least amino acid #377 through amino acid #472. These proteins are capable of inducing cartilage and or bone formation.

Human BMP-3 proteins are produced by culturing a cell transformed with a DNA sequence substantially as shown in Table II and recovering from the culture medium a protein containing substantially the 96 amino acid sequence as shown in Table II from amino acid # 377 through amino acid # 472.

Members of the BMP-3 protein family may be further characterized by the ability to demonstrate cartilage and/or bone formation activity in the rat bone formation assay described below. In preferred embodiments the proteins of the invention demonstrate activity in this rat bone formation assay at a concentration of .5μg - 100μg/gram of bone. In more preferred embodiments these proteins demonstrate activity in this assay at a concentration of 1μg - 50μg/gram of bone. More particularly, these proteins may be characterized by the ability of 1μg of the protein to score at least +2 in the rat bone formation assay.

Another aspect of the invention provides pharmaceutical compositions containing a therapeutically effective amount of a BMP-3 protein in admixture with a pharmaceutically acceptable vehicle or carrier. The compositions may be used for bone

and/or cartilage formation and may also be used for wound healing and tissue repair. Compositions of the invention may further include other therapeutically useful agents such as the BMP proteins BMP-1, BMP-2A, and BMP-2B disclosed in PCT publication WO88/00205. Other therapeutically useful agents include growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), and transforming growth factors (TGF-α and TGF-β). The compositions may also include an appropriate matrix, for instance, for supporting the compositions and providing a surface for bone and/or cartilage growth.

The compositions may be employed in methods for treating a number of bone defects and periodontal disease and various types of wounds. These methods, according to the invention, entail administering to a patient needing such bone and/or cartilage formation, wound healing, or tissue repair an effective amount of a novel BMP-3 protein of the present invention. These methods may also entail the administration of a BMP-3 protein of the invention in conjunction with at least one of the novel BMP proteins disclosed in PCT publication WO88/00205. In addition, these methods may also include administration of a BMP-3 with other growth factors.

Still a further aspect of the invention are DNA sequences coding for expression of a BMP-3 protein. Such sequences include the sequence of nucleotides in a 5' to 3' direction illustrated in Tables I A and I B and II or DNA sequences which hybridize under stringent conditions with the DNA sequences of Tables I A and I B and II and encode a protein having the ability to induce cartilage and/or bone formation. It is preferred that such proteins be further characterized by the ability to demonstrate cartilage and/or bone formation activity in the rat bone formation assay described below. In preferred embodiments the proteins of the invention demonstrate activity in this rat bone formation assay at a concentration of .5μg - 100μg/gram of bone. In more preferred embodiments

these proteins demonstrate activity in this assay at a concentration of 1μg - 50μg/gram of bone. More particularly, these proteins may be characterized by the ability of 1μg of the protein to score at least +2 in the rat bone formation assay. Finally, allelic or other variations of the sequences of Tables I A and I B and II, whether such nucleotide changes result in changes in the peptide sequence or not, are also included in the present invention.

Still a further aspect of the invention is a vector containing a DNA sequence as described above in operative association with an expression control sequence therefor. Such vector may be employed in a novel process for producing a BMP-3 protein of the invention in which a cell line transformed with a DNA sequence encoding expression of a BMP-3 protein in operative association with an expression control sequence therefor, is cultured in a suitable culture medium and a BMP-3 protein is isolated and purified therefrom. This claimed process may employ a number of known cells both prokaryotic and eukaryotic as host cells for expression of the polypeptide.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description and preferred embodiments thereof.

Detailed Description of the Invention

The purified BMP-3 proteins of the present invention are produced by culturing a host cell transformed with a DNA sequence comprising substantially as shown in Table II from nucleotide #321 to nucleotide #1736 or a portion thereof and recovered from the culture medium. The recovered BMP-3 proteins are characterized by the 96 amino acid sequence of a substantially homologous sequence as amino acid # 377 to amino acid # 472 as shown in Table II. These proteins may be further characterized by the ability to demonstrate cartilage and/or bone formation activity in the rat bone

formation assay described below. In preferred embodiments they demonstrate activity in this rat bone formation assay at a concentration of .5μg - 100μg/gram of bone. In more preferred embodiments these proteins demonstrate activity in this assay at a concentration of 1μg - 50μg/gram of bone. More particularly, these proteins may be characterized by the ability of 1μg of the protein to score at least +2 in the rat bone formation assay. Encompassed within the BMP-3 family of proteins of the invention are multiple variant forms including dimers and monomers both precursor and mature forms.

The BMP-3 proteins provided herein also include proteins encoded by the sequences similar to those of Tables I A and I B and II, but into which modifications are naturally provided (e.g. allelic variations in the nucleotide sequence which may result in amino acid changes in the polypeptide) or deliberately engineered. For example, synthetic polypeptides may wholly or partially duplicate continuous sequences of the amino acid residues of Tables I A and I B and II. These sequences, by virtue of sharing primary, secondary, or tertiary structural and conformational characteristics with BMP-3 proteins of Tables I A and I B and II may possess biological properties in common therewith. Thus, they may be employed as biologically active substitutes for naturally-occurring BMP-3 polypeptides in therapeutic processes.

Other specific mutations of the sequences of BMP-3 described herein involve modifications of the glycosylation sites. The absence of glycosylation or only partial glycosylation results from amino acid substitution or deletion at one or both of the asparagine-linked glycosylation recognition sites present in the sequences of the BMP-3 shown in Tables I A and I B and II. The asparagine-linked glycosylation recognition sites comprise tripeptide sequences which are specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either asparagine-X-threonine or asparagine-X-serine, where X is

usually any amino acid. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence.

The present invention also encompasses the novel DNA sequences, free of association with DNA sequences encoding other proteinaceous materials, and coding on expression for a BMP-3 protein. These DNA sequences include those depicted in Tables I A and I B and II in a 5' to 3' direction and those sequences which hybridize under stringent hybridization conditions [see, T. Maniatis et al, Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory (1982), pages 387 to 389] to the DNA sequences of Tables I A and I B and II and demonstrate cartilage and/or bone formation activity. An example of one such stringent hybridization condition is hybridization at 4X SSC at 65°C, followed by a washing in 0.1 X SSC at 65°C for an hour. Alternatively, an exemplary stringent hybridization condition is in 50% formamide, 4 X SCC at 42°C.

Similarly, DNA sequences which code for a BMP-3 polypeptides coded for by the sequences of Tables I A and I B and II, but which differ in codon sequence due to the degeneracies of the genetic code or allelic variations (naturally-occurring base changes in the species population which may or may not result in an amino acid change) also encode the novel growth factors described herein. Variations in the DNA sequences of Tables I A and I B and II which are caused by point mutations or by induced modifications (including insertion, deletion, and substitution) to enhance the activity, half-life or production of the polypeptides encoded thereby are also encompassed in the invention.

Another aspect of the present invention provides a novel method for producing BMP-3 proteins. The method of the present invention involves culturing a suitable cell or cell

line, which has been transformed with a DNA sequence coding on expression for a BMP-3 polypeptide of the invention, under the control of known regulatory sequences recovering and purifying the proteins from the culture medium. Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO). The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981), or alternatively, Kaufman et al, Mol. Cell. Biol., 5(7):1750-1759 (1985) or Howley et al, U.S. Patent 4,419,446. Another suitable mammalian cell line, which is described in the accompanying examples, is the monkey COS-1 cell line. The mammalian cell line CV-1 may also be useful.

Bacterial cells may also be suitable hosts. For example, the various strains of E. coli (e.g., HB101, MC1061) are well-known as host cells in the field of biotechnology. Various strains of B. subtilis, Pseudomonas, other bacilli and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art may also be available as host cells for expression of the polypeptides of the present invention. Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention. See, e.g. Miller et al, Genetic Engineering, 8:277-298 (Plenum Press 1986) and references cited therein.

Another aspect of the present invention provides vectors for use in the method of expression of these novel BMP-3 polypeptides. Preferably the vectors contain the full novel DNA sequences described above which code for the novel BMP-3 factors of the invention. Additionally the vectors also contain appropriate expression control sequences permitting expression of the BMP-3 protein sequences. Alternatively, vectors incorporating modified sequences as described above are also

embodiments of the present invention and useful in the production of the BMP-3 proteins. The vectors may be employed in the method of transforming cell lines and contain selected regulatory sequences in operative association with the DNA coding sequences of the invention which are capable of directing the replication and expression thereof in selected host cells. Useful regulatory sequences for such vectors are known to one of skill in the art and may be selected depending upon the selected host cells. Such selection is routine and does not form part of the present invention. Host cells transformed with such vectors and progeny thereof for use in producing BMP-3 proteins of the invention are also provided by the invention. Furthermore, proteins of the invention may be coexpressed with other "BMP" proteins such as those disclosed in WO88/00205.

A protein of the present invention, which induces cartilage and/or bone growth in circumstances where bone is not normally formed, has application in the healing of bone fractures and cartilage defects in humans and other animals. Such a preparation employing a BMP-3 protein may have prophylactic use in closed as well as open fracture reduction and also in the improved fixation of artificial joints. BMP-3 preparations of the invention may also be useful in the treatment of osteoporosis. De novo bone formation induced by an osteogenic agent contributes to the repair of congenital, trauma induced, or oncologic resection induced craniofacial defects, and also is useful in cosmetic plastic surgery. A BMP-3 protein of the invention may be valuable in the treatment of periodontal disease, and in other tooth repair processes. Such agents may provide an environment to attract bone-forming cells, stimulate growth of bone-forming cells or induce differentiation of progenitors of bone-forming cells. A variety of osteogenic, cartilage-inducing and bond inducing factors have been described. See, e.g. European patent applications 148,155 and 169,016 for discussions thereof.

The proteins of the invention may also be used in wound healing and tissue repair in humans and other animals. The types of wounds include, but are not limited to burns, incisions, and ulcers. (See, e.g., PCT Publication WO84/01106 for discussion of wound healing and related tissue repair). Of course, the proteins of the invention may have other therapeutic uses.

A further aspect of the invention is a therapeutic method and composition for repairing fractures and other conditions related to cartilage and/or bone defects or periodontal diseases. In addition, the invention comprises therapeutic methods and compositions for wound healing and tissue repair. Such compositions comprise a therapeutically effective amount of a BMP-3 protein in admixture with a pharmaceutically acceptable vehicle, carrier or matrix. It is expected that BMP-3 proteins may act in concert with or perhaps synergistically with other related proteins and growth factors. The invention encompasses therapeutic methods and compositions comprising a BMP-3 protein in combination with other related proteins or growth factors. Therapeutic methods and compositions of the invention may therefore comprise a therapeutic amount of a BMP-3 protein with a therapeutic amount of at least one of the other "BMP" proteins disclosed in PCT publication WO88/00205. Such combinations may comprise separate molecules of the "BMP" proteins or heteromolecules comprised of different "BMP" protein moieties. For example, a method and composition of the invention may comprise a disulfide-linked dimer comprising a BMP-3 protein and another "BMP" protein described above. Further, a BMP-3 protein of the invention may be combined with other agents beneficial to the treatment of the cartilage and/or bone defect, wound or tissue in question. These agents include various growth factors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), transforming growth factors (TGF-α and TGF-β), insulin-like growth factor (IGF) and

fibroblast growth factor (FGF). The preparation and formulation of such physiologically acceptable protein compositions, having due regard to pH, isotonicity, stability and the like, is within the skill of the art. The therapeutic compositions of the invention are also presently valuable for veterinary applications due to the lack of species specificity in BMP proteins. Particularly domestic animals and thoroughbred horses in addition to humans are desired patients for such treatment with BMP-3 proteins.

The therapeutic method includes administering the composition topically, systematically, or locally as an implant or device. When administered, the therapeutic composition for use in this invention is, of course, in a pyrogen-free, physiologically acceptable form. Further, the composition may desirably be encapsulated or injected in a viscous form for delivery to the site of bone, cartilage or tissue damage. Topical administration may be suitable for wound healing and related tissue repair. Preferably, for bone and/or cartilage formation, the bone growth inductive factor composition would include a matrix capable of delivering the bone inductive factor to the site of bone and/or cartialge damage, providing a surface and support structure for the developing bone and/or cartilage and optimally capable of being resorbed into the body. Such matrices may be formed of materials presently in use for other implanted medical applications.

The choice of matrix material is based on, for example, biocompatibility, biodegradability, mechanical properties, cosmetic appearance and interface properties. The particular application of the BMP-3 compositions will determine the appropriate formulation. Potential matrices for the compositions may be biodegradable and chemically defined such as calcium sulfate, tricalciumphosphate, hydroxyapatite, polylactic acid, and polyanhydrides. Other potential materials are biodegradable and biologically well defined, such as bone

or dermal collagen. Further matrices are comprised of pure proteins or extracellular matrix components. Other potential matrices are nonbiodegradable and chemically defined, such as sintered hydroxyapatite, bioglass, aluminates, or other ceramics. Matrices may be comprised of combinations of any of the above mentioned types of material, such as polylactic acid and hydroxyapatite or collagen and tricalciumphosphate. The bioceramics may also be altered in composition, such as in calcium-aluminate-phosphate and processing to alter for example, pore size, particle size, particle shape, and biodegradability.

The dosage regimen will be determined by the attending physician considering various factors which modify the action of the BMP-3 protein, e.g. amount of bone weight desired to be formed, the site of bone damage, the condition of the damaged bone, the size of a wound, type of damaged tissue, the patient's age, sex, and diet, the severity of any infection, time of administration and other clinical factors. The dosage may vary with the type of matrix used in the reconstitution and the types of BMP proteins in the composition. The addition of other known growth factors, such as IGF-I (insulin like growth factor I), to the final composition, may also effect the dosage.

Generally, the dosage regimen for cartilage and/or bone formation should be in the range of approximately 10 to 10^6 nanograms of protein per gram of bone weight desired. Progress can be monitored by periodic assessment of bone growth and/or repair, for example, using x-rays, histomorphometric determinations and tetracycline labeling.

The following examples illustrate practice of the present invention in recovering and characterizing a bovine BMP-3 protein and employing it to recover corresponding human BMP-3 proteins, and in expressing BMP-3 proteins via recombinant techniques.

EXAMPLE I

Isolation of Bovine Bone Inductive Factor

Ground bovine bone powder (20-120 mesh, Helitrex) is prepared according to the procedures of M. R. Urist et al., Proc. Natl Acad. Sci USA, 70:3511 (1973) with elimination of some extraction steps as identified below. Ten kgs of the ground powder is demineralized in successive changes of 0.6N HCl at 4°C over a 48 hour period with vigorous stirring. The resulting suspension is extracted for 16 hours at 4°C with 50 liters of 2M $CaCl_2$ and 10mM ethylenediamine-tetraacetic acid [EDTA], and followed by extraction for 4 hours in 50 liters of 0.5M EDTA. The residue is washed three times with distilled water before its resuspension in 20 liters of 4M guanidine hydrochloride [GuCl], 20mM Tris (pH 7.4), 1mM N-ethylmaleimide, 1mM iodoacetamide, 1mM phenylmethylsulfonyl fluorine as described in Clin. Orthop. Rel. Res., 171: 213 (1982). After 16 to 20 hours the supernatant is removed and replaced with another 10 liters of GuCl buffer. The residue is extracted for another 24 hours.

The crude GuCl extracts are combined, concentrated approximately 20 times on a Pellicon apparatus with a 10,000 molecular weight cut-off membrane, and then dialyzed in 50mM Tris, 0.1M NaCl, 6M urea (pH7.2), the starting buffer for the first column. After extensive dialysis the protein is loaded on a 4 liter DEAE cellulose column and the unbound fractions are collected.

The unbound fractions are concentrated and dialyzed against 50mM NaAc, 50mM NaCl (pH 4.6) in 6M urea. The unbound fractions are applied to a carboxymethyl cellulose column. Protein not bound to the column is removed by extensive washing with starting buffer, and the material containing protein having bone and/or cartilage formation activity as measured by the Rosen-modified Sampath-Reddi rat bone formation assay (described in Example III below) is desorbed from the column by 50mM NaAc, 0.25mM NaCl, 6M urea (pH 4.6). The protein from this

step elution is concentrated 20- to 40- fold, then diluted 5 times with 80mM KPO_4, 6M urea (pH6.0). The pH of the solution is adjusted to 6.0 with 500mM K_2HPO_4. The sample is applied to an hydroxylapatite column (LKB) equilibrated in 80mM KPO_4, 6M urea (pH6.0) and all unbound protein is removed by washing the column with the same buffer. Protein having bone and/or cartilage formation activity as measured by the rat bone formation assay is eluted with 100mM KPO_4 (pH7.4) and 6M urea.

The protein is concentrated approximately 10 times, and solid NaCl added to a final concentration of 0.15M. This material is applied to a heparin - Sepharose column equilibrated in 50mM KPO_4, 150mM NaCl, 6M urea (pH7.4). After extensive washing of the column with starting buffer, a protein with bone and/or cartilage formation activity is eluted by 50mM KPO_4, 700mM NaCl, 6M urea (pH7.4). This fraction is concentrated to a minimum volume, and 0.4ml aliquots are applied to Superose 6 and Superose 12 columns connected in series, equilibrated with 4M GuCl, 20mM Tris (pH7.2) and the columns developed at a flow rate of 0.25ml/min. The protein demonstrating bone and/or cartilage inductive activity has a relative migration on SDS-PAGE corresponding to an approximately 28,000 to 30,000 dalton protein.

The above fractions from the superose columns are pooled, dialyzed against 50mM NaAc, 6M urea (pH4.6), and applied to a Pharmacia MonoS HR column. The column is developed with a gradient to 1.0M NaCl, 50mM NaAc, 6M urea (pH4.6). Active fractions are pooled and brought to pH3.0 with 10% trifluoroacetic acid (TFA). The material is applied to a 0.46 x 25cm Vydac C4 column in 0.1% TFA and the column developed with a gradient to 90% acetonitrile, 0.1% TFA (31.5% acetonitrile, 0.1% TFA to 49.5% acetonitrile, 0.1% TFA in 60 minutes at 1ml per minute). Active bone and/or cartilage forming material is eluted at approximately 40-44% acetonitrile. Aliquots of the appropriate active fractions are iodinated by one of the following methods: P. J. McConahey et

al, Int. Arch. Allergy, 29:185-189 (1966); A. E. Bolton et al, Biochem J., 133:529 (1973); and D. F. Bowen-Pope, J. Biol. Chem., 237:5161 (1982). The iodinated proteins present in these fractions are analyzed by SDS gel electrophoresis and urea Triton X 100 isoelectric focusing. At this stage, the bone inductive factor is estimated to be approximately 10-50% pure.

EXAMPLE II

Characterization of Bovine Bone Inductive Factor

A. Molecular Weight

Approximately 20ug protein from Example I is lyophilized and redissolved in 1X SDS sample buffer. After 15 minutes of heating at 37°C, the sample is applied to a 15% SDS polyacrylamide gel and then electrophoresed with cooling. The molecular weight is determined relative to prestained molecular weight standards (Bethesda Research Labs). Immediately after completion, the gel lane containing the bone and/or cartilage forming material is sliced into 0.3cm pieces. Each piece is mashed and 1.4ml of 0.1% SDS is added. The samples are shaken gently overnight at room temperature to elute the protein. Each gel slice is desalted to prevent interference in the biological assay. The supernatant from each sample is acidified to pH 3.0 with 10% TFA, filtered through a 0.45 micron membrane and loaded on a 0.46cm x 5cm C4 Vydac column developed with a gradient of 0.1% TFA to 0.1% TFA, 90% CH_3CN. The appropriate bone and/or cartilage inductive protein - containing fractions are pooled and reconstituted with 20mg rat matrix and assayed. In this gel system, the majority of bone and/or cartilage formation fractions have the mobility of a protein having a molecular weight of approximately 28,000 - 30,000 daltons.

B. Isoelectric Focusing

The isoelectric point of the protein having bone and/or

cartilage formation activity is determined in a denaturing isoelectric focusing system. The Triton X100 urea gel system (Hoeffer Scientific) is modified as follows: 1) 40% of the ampholytes used are Servalyte 3/10; 60% are Servalyte 7-9; and 2) the catholyte used is 40mM NaOH. Approximately 20ug of protein from Example I is lyophilized, dissolved in sample buffer and applied to the isoelectrofocusing gel. The gel is run at 20 watts, 10°C for approximately 3 hours. At completion the lane containing bone and/or cartilage inductive factor is sliced into 0.5 cm slices. Each piece is mashed in 1.0ml 6M urea, 5mM Tris (pH 7.8) and the samples agitated at room temperature. The samples are acidified, filtered, desalted and assayed as described above. The major portion of activity as determined by the Rosen-modified Sampath-Reddi assay migrates in a manner consistent with a pI of about 8.8 - 9.2.

C. Subunit Characterization

The subunit composition of the isolated bovine bone protein is also determined. Pure bone inductive factor is isolated from a preparative 15% SDS gel as described above. A portion of the sample is then reduced with 5mM DTT in sample buffer and re-electrophoresed on a 15% SDS gel. The approximately 28-30kd protein yields two major bands at approximately 18 - 20kd and approximately 16 - 18kd, as well as a minor band at approximately 28 - 30kd. The broadness of the two bands indicates heterogeneity caused most probably by glycosylation, other post translational modification, proteolytic degradation or carbamylation.

EXAMPLE III

Rosen Modified Sampath-Reddi Assay

A modified version of the rat bone formation assay described in Sampath and Reddi, Proc. Natl. Acad. Sci. U.S.A., 80:6591-6595 (1983) is used to evaluate bone and/or cartilage activity of the bovine protein obtained in Example I and the

BMP-1 proteins of the invention. This modified assay is herein called the Rosen-modified Sampath-Reddi assay. The ethanol precipitation step of the Sampath-Reddi procedure is replaced by dialyzing (if the composition is a solution) or diafiltering (if the composition is a suspension) the fraction to be assayed against water. The solution or suspension is then redissolved in 0.1 % TFA, and the resulting solution added to 20mg of rat matrix. A mock rat matrix sample not treated with the protein serves as a control. This material is frozen and lyophilized and the resulting powder enclosed in #5 gelatin capsules. The capsules are implanted subcutaneously in the abdominal thoracic area of 21 - 49 day old male Long Evans rats. The implants are removed after 7 - 14 days. Half of each implant is used for alkaline phosphatase analysis [See, A. H. Reddi et al., Proc. Natl Acad Sci., 69:1601 (1972)].

The other half of each implant is fixed and processed for histological analysis. 1um glycolmethacrylate sections are stained with Von Kossa and acid fuschin to score the amount of induced bone and cartilage formation present in each implant. The terms +1 through +5 represent the area of each histological section of an implant occupied by new bone and/or cartilage cells and matrix. A score of +5 indicates that greater than 50% of the implant is new bone and/or cartilage produced as a direct result of protein in the implant. A score of +4, +3, +2 and +1 would indicate that greater than 40%, 30%, 20% and 10% respectively of the implant contains new cartilage and/or bone.

The rat matrix samples containing 200 ng of protein obtained in Example I result in bone and/or cartilage formation that filled more than 20% of the implant areas that was sectioned for histology. This protein therefore scores at least +2 in the Rosen-modified Sampath-Reddi assay. The dose response of the matrix samples indicates that the amount of bone and/or cartilage formed increases with the amount of protein in the sample. The control sample did not result in

any bone and/or cartilage formation. The purity of the protein assayed is approximately 10-15% pure.

The bone and/or cartilage formed is physically confined to the space occupied by the matrix. Samples are also analyzed by SDS gel electrophoresis and isoelectric focusing as described above, followed by autoradiography. Analysis reveals a correlation of activity with protein bands at 28 - 30kd and a pI of approximately 8.8 - 9.2. To estimate the purity of the protein in a particular fraction an extinction coefficient of 1 OD/mg-cm is used as an estimate for protein and the protein is run on SDS PAGE followed by silver staining or radioiodination and autoradiography.

EXAMPLE IV

Bovine BMP-3

The protein composition of Example IIA of molecular weight 28 - 30kd is reduced in situ and digested with trypsin. Eight tryptic fragments are isolated by standard procedures having the following amino acid sequences:

Fragment 1: A A F L G D I A L D E E D L G
Fragment 2: A F Q V Q Q A A D L
Fragment 3: N Y Q D M V V E G
Fragment 4: S T P A Q D V S R
Fragment 5: N Q E A L R
Fragment 6: L S E P D P S H T L E E
Fragment 7: F D A Y Y
Fragment 8: L K P S N ? A T I Q S I V E

A less highly purified preparation of protein from bovine bone is prepared according to a purification scheme similar to that described in Example I. The purification basically varies from that previously described by omission of the DE-52 column, the CM cellulose column and the mono S column, as well as a reversal in the order of the hydroxylapatite and heparin sepharose columns. Briefly, the concentrted crude 4 M extract is brought to 85% final

concentration of ethanol at 4 degrees. The mixture is then centrifuged, and the precipitate redissolved in 50 mM Tris, 0.15 M NaCl, 6.0 M urea. This material is then fractionated on Heparin Sepharose as described. The Heparin bound material is fractionated on hydroxyapatite as described. The active fractions are pooled, concentrated, and fractionated on a high resolution gel filtration (TSK 30000 in 6 M guanidinium chloride, 50 mM Tris, pH 7.2). The active fractions are pooled, dialyzed against 0.1% TFA, and then fractionated on a C4 Vydac reverse phase column as described. A small amount of ^{125}I labeled counterpart is mixed with the sample at this stage and the whole preparation is reduced and electrophoresed on an SDS ployacrylanide acrylamide gel [Laemmli, U.K., Nature, 277:680-685 (1970)]. The protein corresponding to the 16-18kd band is located using wet gel autoradiography and fixed with methanol-acetic acid-water using standard procedures, briefly rinsed with water, then neutralized with 0.1M ammonium bicarbonate. Following dicing the gel slice with a razor blade, the protein is digested from the gel matrix by adding 0.2 μg of TPCK-treated trypsin (Worthington) and incubating the gel for 16 hours at 37°C. The resultant digest is then subjected to RPHPLC using a C4 Vydac RPHPLC column and 0.1% TFA-water 0.1% TFA water-acetonitrile gradient. The resultant peptide peaks were monitored by UV absorbance at 214 and 280 nm and subjected to direct amino terminal amino acid sequence analysis using an Applied Biosystems gas phase sequenator (Model 470A). Tryptic fragments are isolated having the following amino acid sequences:

Fragment 9: S L K P S N H A T I Q S ? V

Fragment 10: S F D A Y Y C S ? A

Fragment 11: V Y P N M T V E S C A

Fragment 12: V D F A D I ? W

Tryptic Fragments 7 and 8 are noted to be substantially the same as Fragments 10 and 9, respectively.

Probes consisting of pools of oligonucleotides (or unique oligonucleotides) are designed on the basis of the amino acid sequences of the tryptic Fragments 9 (Probe #3), 10 (Probe #2), and 11 (Probe #1), according to the method of R. Lathe, J. Mol. Biol., 183(1): 1-12 (1985), and synthesized on an automated DNA synthesizer; the probes are then radioactively labeled with polynucleotide Kinase and ^{32}P-ATP.

Probe #1: A C N G T C A T [A/G] T T N G G [A/G] T A

Probe #2: C A [A/G] T A [A/G] T A N G C [A/G] T C [A/G] A A

Probe #3: T G [A/G/T] A T N G T N G C [A/G] T G [A/G] T T

The standard nucleotide symbols in the above-identified probes are as follows: A, adenosine; C, cytosine; G, guanine; T, thymine; and N, adenosine or cytosine or guanine or thymine.

Because the genetic code is degenerate (more than one codon can code for the same amino acid), the number of oligonucleotides in a probe pool is reduced based on the frequency of codon usage in eukaryotes, the relative stability of G:T base pairs, and the relative infrequency of the dinucleotide CpG in eukaryotic coding sequences [See Toole et al., Nature, 312:342-347 (1984)].

A recombinant bovine genomic library is constructed as follows: Bovine liver DNA is partially digested with the restriction endonuclease enzyme Sau 3A and sedimented through a sucrose gradient. Size fractionated DNA in the range of 15-30kb is then ligated to the bacteriophage Bam HI vector EMBL3 [Frischauf et al, J. Mol. Biol., 170:827-842 (1983)]. The library is plated at 8000 recombinants per plate. Duplicate nitrocellulose replicas of the plaques are made and amplified according to a modification of the procedure of Woo et al, Proc. Natl. Acad. Sci. USA, 75:3688-91 (1978). 400,000 recombinants are screened in duplicate with Probe #1 which has been labeled with ^{32}P. The probes are hybridized in

3M tetramethylammonium chloride (TMAC), 0.1M sodium phosphate pH6.5, 1mM EDTA, 5X Denhardts, 0.6% SDS, 100ug/ml salmon sperm DNA at 48 degrees C, and washed in 3M TMAC, 50mM Tris pH8.0 at 50 degrees C. These conditions minimize the detection of mismatches to the 17 mer probe pool [see, Wood et al, Proc. Natl. Acad. Sci, U.S.A., 82:1585-1588 (1985)]. All recombinants which hybridized to this probe are replated for secondaries. Triplicate nitrocellulose replicas are made of the secondary plates, and amplified as described. The three sets of filters are hybridized to Probes #1, #2 and #3, again under TMAC conditions. One clone, lambda bP-819, hybridizes to all three probes and is plaque purified and DNA is isolated from a plate lysate. Bacteriophage lambda bP-819 was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockland, Maryland USA (hereinafter the ATCC) on June 16, 1987 under accession number 40344. This deposit meets the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and Regulations thereunder. This bP-819 clone encodes at least a portion of the bovine protein which we have designated BMP-3 or bBMP-3.

The region of bP-819 which hybridizes to Probe #2 is localized and sequenced. The partial DNA and derived amino acid sequences of this region are shown in Table IIA. The amino acid sequences corresponding to tryptic Fragments 10 and 12 are underlined. The first underlined sequence corresponds to Fragment 12 while the second corresponds to Fragment 10. This region of bP-819, therefore, which hybridizes to Probe #2 encodes at least 111 amino acids. This amino acid sequence is encoded by the DNA sequence from nucleotide #414 through #746.

TABLE I. A.

```
        383         393         403         413  (I)             428
GAGGAGGAAG CGGTCTACGG GGGTCCTTCT GCCTCTGCAG  AAC AAT GAG CTT CCT GGG GCA
                                             Asn Asn Glu Leu Pro Gly Ala

        443                 458                 473                 488
GAA TAT CAG TAC AAG GAG GAT GAA GTA TGG GAG GAG AGG AAG CCT TAC AAG ACT
Glu Tyr Gln Tyr Lys Glu Asp Glu Val Trp Glu Glu Arg Lys Pro Tyr Lys Thr

                503                 518                 533
CTT CAG ACT CAG CCC CCT GAT AAG AGT AAG AAC AAA AAG AAA CAG AGG AAG GGA
Leu Gln Thr Gln Pro Pro Asp Lys Ser Lys Asn Lys Lys Lys Gln Arg Lys Gly

    548                 563                 578                 593
CCT CAG CAG AAG AGT CAG ACG CTC CAG TTT GAT GAA CAG ACC CTG AAG AAG GCA
Pro Gln Gln Lys Ser Gln Thr Leu Gln Phe Asp Glu Gln Thr Leu Lys Lys Ala

            608                 623                 638
AGA AGA AAG CAA TGG ATT GAA CCC CGG AAT TGT GCC AGA CGG TAC CTT AAA GTG
Arg Arg Lys Gln Trp Ile Glu Pro Arg Asn Cys Ala Arg Arg Tyr Leu Lys Val

653                 668                 683                 698
GAC TTC GCA GAT ATT GGC TGG AGC GAA TGG ATT ATT TCC CCC AAG TCC TTC GAT
Asp Phe Ala Asp Ile Gly Trp Ser Glu Trp Ile Ile Ser Pro Lys Ser Phe Asp

        713                 728                 743 (111)       756
GCC TAT TAC TGC TCC GGA GCG TGC CAG TTC CCC ATG CCA AAG  GTAGCCATTG
Ala Tyr Tyr Cys Ser Gly Ala Cys Gln Phe Pro MET Pro Lys

      766         776         786
TTTTTTGTCC TGTCCTTCCC ATTTCCATAG
```

The region of bP-819 which hybridizes to Probe #1 and #3 is localized and sequenced. The partial DNA and derived amino acid sequences of this region are shown in Table IIB. The amino acid sequences corresponding to tryptic Fragments 9 and 11 are underlined. The first underlined sequence corresponds to Fragment 9 while the second underlined sequence corresponds to Fragment 11. The peptide sequence of this region of bP-819 which hybridizes to Probe #1 and #3 is 64 amino acids in length encoded by nucleotide #305 through #493 of Table IIB. The arginine residue encoded by the AGA triplet is presumed to be the carboxy-terminus of the protein based on the presence of a stop codon (TAA) adjacent to it. The nucleic acid sequence preceding the couplet TC (positions 305-306) is presumed to be an intron (non-coding sequence) based on the presence of a consensus acceptor sequence (i.e. a pyrimidine-rich stretch, TTCTCCCTTTTCGTTCCT, followed by AG) and the presence of a stop rather than a basic residue in the appropriate position of the derived amino acid sequence.

bBMP-3 is therefore characterized by the DNA and amino acid sequence of Table I A and Table I B. The peptide sequence of this clone is 175 amino acids in length and is encoded by the DNA sequence from nucleotide #414 through nucleotide #746 of Table I A and nucleotide #305 through nucleotide #493 of Table I B.

TABLE I. B.

```
       284        294        304  (112)          319
CTAACCTGTG TTCTCCCTTT TCGTTCCTAG  TCT TTG AAG CCA TCA AAT CAC GCT ACC
                                  Ser Leu Lys Pro Ser Asn His Ala Thr

  334                 349                 364                 379
ATC CAG AGT ATA GTG AGA GCT GTG GGG GTC GTC CCT GGA ATC CCC GAG CCT TGC
Ile Gln Ser Ile Val Arg Ala Val Gly Val Val Pro Gly Ile Pro Glu Pro Cys

         394                 409                 424                 439
TGT GTG CCA GAA AAG ATG TCC TCA CTC AGC ATC TTA TTC TTT GAT GAA AAC AAG
Cys Val Pro Glu Lys MET Ser Ser Leu Ser Ile Leu Phe Phe Asp Glu Asn Lys

                454                 469                 484        (175)
AAT GTG GTA CTT AAA GTA TAT CCA AAC ATG ACA GTA GAG TCT TGT GCT TGC AGA
Asn Val Val Leu Lys Val Tyr Pro Asn MET Thr Val Glu Ser Cys Ala Cys Arg

      503        513        523        533
TAACCTGGTG AAGAACTCAT CTGGATGCTT AACTCAATCG
```

EXAMPLE V

Human BMP-3

The bovine and human BMP-3 genes are presumed to be significantly homologous, therefore a human genomic library is screened with two oligonucleotide probes synthesized with the bovine BMP-3 sequence above. The oligonucleotides are as follows

#1: d(AATTCCGGGGTTCAATCCATTGCTTTCTTCTTGCCTTCTTCAGGGTCTCTGT)

#2: d(TTCGCTCCAGCCAATATCTGCGAAGTCCACTTTAAGGTACCGTCTGGCAC)

The oligonucleotides are synthesized on an automated synthesizer and radioactively labeled with polynucleotide kinase and ^{32}p-ATP. A human genomic library (Toole et al., supra) is plated. Duplicate nitrocellulose filter replicas of the library corresponding to 1,000,000 recombinants are made of and hybridized to the nick-translated probes in 5 X SSC, 5 X Denhardt's, 100ug/ml denatured salmon sperm DNA, 0.1% SDS (the standard hybridization solution) at 50 degrees centigrade for approximately 14 hours. The filters are then washed in 1 X SSC, 0.1% SDS at 50°C and subjected to autoradiography. Ten duplicate positives are isolated and plaque purified. Sequence analysis indicates that the positives contain the human BMP-3 gene.

A region comprised of the bovine DNA sequence residues 408-727 in Table I.A. is subcloned into the plasmid pSP65 [see D.A. Melton et al, Nucl. Acid Res., 12:7035-7056 (1984)], and amplified by standard techniques. The insert region of this plasmid is then excised and labeled with ^{32}p by nick-translation. A primer-extended cDNA library is made from the human lung small cell carcinoma cell line H128 (ATCC# HTB 120) using as a primer an oligonucleotide of the sequence d(AATGATTGAATTAAGCAATTC). This oligonucleotide was synthesized on the basis of the DNA sequence of the 3' untranslated region of the human BMP-3 gene. 375,000 recombinants from this library are screened with the nick-translated probe by standard methods. Recombinants from the library are hybridized

to the probe in standard hybridization solution at 65 and washed in 0.2 x SSc, 0.1% SDS at 65°C. 17 positives are obtained. One of these, λH128-4 was deposited with the ATCC on March 31, 1988 under accession number 40437. This deposit meets the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and Regulations thereunder. The entire nucleotide sequence and derived amino acid sequence of the insert of H128-4 are given in Table II. This clone is expected to contain all of the nucleotide sequence necessary to encode the entire BMP-3 protein. The amino acid sequence of Table II is contemplated to represent a primary translation product which may be cleaved to produce the mature protein/s. Nucleotide #1 to #320 represents the 5' untranslated region and nucleotide #1736 to #1794 represents the 3' untranslated region. Precursor proteins may be cleaved at the proteolytic processing site between amino acid #360 and #361. The BMP-3 proteins encoded by Table II are contemplated to contain the 96 amino acid sequence from amino acid #377 to amino acid #472 or a sequence substantially homologous thereto. The sequences corresponding to tryptic Fragments 9-12 are underlined in Table II. The DNA sequence indicates that the human BMP-3 precursor protein is 472 amino acids. It is contemplated that BMP-3 corresponds to the approximately 16 to 18 kd subunit of Example IIC.

The sequences of BMP-3 as shown in Tables I A and I B and II, have significant homology to the beta (B) and beta (A) subunits of the inhibins. The inhibins are a family of hormones which are presently being investigated for use in contraception. See, A. J. Mason et al, Nature, 318:659-663 (1985). To a lesser extent they are also homologous to Mullerian inhibiting substance (MIS), a testicular glycoprotein that causes regression of the Mullerian duct during development of the male embryo and transforming growth factor-beta (TGF-b) which can inhibit or stimulate growth of cells or cause

them to differentiate. BMP-3 also demonstrates sequence similarity with Vgl. Vgl mRNA has been localized to the vegetal hemisphere of xenopus oocytes. During early development it is distributed throughout the endoderm, but the mRNA is not detectable after blastula formation has occurred. The Vgl protein may be the signal used by the endoderm cells to commit ectodermal cells to become the embryonic mesoderm. BMP-3 also shares some sequence similarity with the bone inductive protein BMP-2A disclosed in PCT publication WO88/00205.

TABLE II

```
          10         20         30         40         50         60         70
AGATCTTGAA AACACCCGGG CCACACACGC CGCGACCTAC AGCTCTTTCT CAGCGTTGGA GTGGAGACGG

          80         90        100        110        120        130        140
CGCCCGCAGC GCCCTGCGCG GGTGAGGTCC GCGCAGCTGC TGGGGAAGAG CCCACCTGTC AGGCTGCGCT

         150        160        170        180        190        200        210
GGGTCAGCGC AGCAAGTGGG GCTGGCCGCT ATCTGCTGC ACCCGGCCGC GTCCCGGGCT CCGTGCGCCC

         220        230        240        250        260        270        280
TCGCCCCAGC TGGTTTGGAG TTCAACCCTC GGCTCCGCCG CCGGCTCCTT GCGCCTTCGG AGTGTCCCGC

         290        300        310        320 (1)            335
AGCGACGCCG GGAGCCGACG CGCCGCGCGG GTACCTAGCC ATG GCT GGG GCG AGC AGG CTG CTC
                                               MET Ala Gly Ala Ser Arg Leu Leu

    350                365                380                395
TTT CTG TGG CTG GGC TGC TTC TGC GTG AGC CTG GCG CAG GGA GAG AGA CCG AAG CCA
Phe Leu Trp Leu Gly Cys Phe Cys Val Ser Leu Ala Gln Gly Glu Arg Pro Lys Pro

        410                425                440                455
CCT TTC CCG GAG CTC CGC AAA GCT GTG CCA GGT GAC CGC ACG GCA GGT GGT GGC CCG
Pro Phe Pro Glu Leu Arg Lys Ala Val Pro Gly Asp Arg Thr Ala Gly Gly Gly Pro

            470                485                500                515
GAC TCC GAG CTG CAG CCG CAA GAC AAG GTC TCT GAA CAC ATG CTG CGG CTC TAT GAC
Asp Ser Glu Leu Gln Pro Gln Asp Lys Val Ser Glu His MET Leu Arg Leu Tyr Asp

                530                545                560
AGG TAC AGC ACG GTC CAG GCG GCC CGG ACA CCG GGC TCC CTG GAG GGA GGC TCG CAG
Arg Tyr Ser Thr Val Gln Ala Ala Arg Thr Pro Gly Ser Leu Glu Gly Gly Ser Gln

575                590                605                620
CCC TGG CGC CCT CGG CTC CTG CGC GAA GGC AAC ACG GTT CGC AGC TTT CGG GCG GCA
Pro Trp Arg Pro Arg Leu Leu Arg Glu Gly Asn Thr Val Arg Ser Phe Arg Ala Ala

    635                650                665                680
GCA GCA GAA ACT CTT GAA AGA AAA GGA CTG TAT ATC TTC AAT CTG ACA TCG CTA ACC
Ala Ala Glu Thr Leu Glu Arg Lys Gly Leu Tyr Ile Phe Asn Leu Thr Ser Leu Thr

        695                710                725                740
AAG TCT GAA AAC ATT TTG TCT GCC ACA CTG TAT TTC TGT ATT GGA GAG CTA GGA AAC
Lys Ser Glu Asn Ile Leu Ser Ala Thr Leu Tyr Phe Cys Ile Gly Glu Leu Gly Asn
```

```
                755                 770                 785                 800
   ATC AGC CTG AGT TGT CCA GTG TCT GGA GGA TGC TCC CAT CAT GCT CAG AGG AAA CAC
   Ile Ser Leu Ser Cys Pro Val Ser Gly Gly Cys Ser His His Ala Gln Arg Lys His
 5
                    815                 830                 845
   ATT CAG ATT GAT CTT TCT GCA TGG ACC CTC AAA TTC AGC AGA AAC CAA AGT CAA CTC
   Ile Gln Ile Asp Leu Ser Ala Trp Thr Leu Lys Phe Ser Arg Asn Gln Ser Gln Leu

10 860                 875                 890                 905
   CTT GGC CAT CTG TCA GTG GAT ATG GCC AAA TCT CAT CGA GAT ATT ATG TCC TGG CTG
   Leu Gly His Leu Ser Val Asp MET Ala Lys Ser His Arg Asp Ile MET Ser Trp Leu

       920                 935                 950                 965
15 TCT AAA GAT ATC ACT CAA TTC TTG AGG AAG GCC AAA GAA AAT GAA GAG TTC CTC ATA
   Ser Lys Asp Ile Thr Gln Phe Leu Arg Lys Ala Lys Glu Asn Glu Glu Phe Leu Ile

           980                 995                1010                1025
   GGA TTT AAC ATT ACG TCC AAG GGA CGC CAG CTG CCA AAG AGG AGG TTA CCT TTT CCA
20 Gly Phe Asn Ile Thr Ser Lys Gly Arg Gln Leu Pro Lys Arg Arg Leu Pro Phe Pro

              1040                1055                1070                1085
   GAG CCT TAT ATC TTG GTA TAT GCC AAT GAT GCC GCC ATT TCT GAG CCA GAA AGT GTG
   Glu Pro Tyr Ile Leu Val Tyr Ala Asn Asp Ala Ala Ile Ser Glu Pro Glu Ser Val
25
                  1100                1115                1130
   GTA TCA AGC TTA CAG GGA CAC CGG AAT TTT CCC ACT GGA ACT GTT CCC AAA TGG GAT
   Val Ser Ser Leu Gln Gly His Arg Asn Phe Pro Thr Gly Thr Val Pro Lys Trp Asp

301145                1160                1175                1190
   AGC CAC ATC AGA GCT GCC CTT TCC ATT GAG CGG AGG AAG AAG CGC TCT ACT GGG GTC
   Ser His Ile Arg Ala Ala Leu Ser Ile Glu Arg Arg Lys Lys Arg Ser Thr Gly Val

      1205                1220                1235                1250
35 TTG CTG CCT CTG CAG AAC AAC GAG CTT CCT GGG GCA GAA TAC CAG TAT AAA AAG GAT
   Leu Leu Pro Leu Gln Asn Asn Glu Leu Pro Gly Ala Glu Tyr Gln Tyr Lys Lys Asp

           1265                1280                1295                1310
   GAG GTG TGG GAG GAG AGA AAG CCT TAC AAG ACC CTT CAG GCT CAG GCC CCT GAA AAG
40 Glu Val Trp Glu Glu Arg Lys Pro Tyr Lys Thr Leu Gln Ala Gln Ala Pro Glu Lys

              1325                1340                1355                1370
   AGT AAG AAT AAA AAG AAA CAG AGA AAG GGG CCT CAT CGG AAG AGC CAG ACG CTC CAA
   Ser Lys Asn Lys Lys Lys Gln Arg Lys Gly Pro His Arg Lys Ser Gln Thr Leu Gln
45
                  1385                1400                1415
   TTT GAT GAG CAG ACC CTG AAA AAG GCA AGG AGA AAG CAG TGG ATT GAA CCT CGG AAT
   Phe Asp Glu Gln Thr Leu Lys Lys Ala Arg Arg Lys Gln Trp Ile Glu Pro Arg Asn

501430                1445    (377)       1460                1475
   TGC GCC AGG AGA TAC CTC AAG GTA GAC TTT GCA GAT ATT GGC TGG AGT GAA TGG ATT
   Cys Ala Arg Arg Tyr Leu Lys Val Asp Phe Ala Asp Ile Gly Trp Ser Glu Trp Ile
```

```
   1490                1505                1520                1535
ATC TCC CCC AAG TCC TTT GAT GCC TAT TAT TGC TCT GGA GCA TGC CAG TTC CCC ATG
Ile Ser Pro Lys Ser Phe Asp Ala Tyr Tyr Cys Ser Gly Ala Cys Gln Phe Pro MET

      1550                1565                1580                1595
CCA AAG TCT TTG AAG CCA TCA AAT CAT GCT ACC ATC CAG AGT ATA GTG AGA GCT GTG
Pro Lys Ser Leu Lys Pro Ser Asn His Ala Thr Ile Gln Ser Ile Val Arg Ala Val

          1610                1625                1640                1655
GGG GTC GTT CCT GGG ATT CCT GAG CCT TGC TGT GTA CCA GAA AAG ATG TCC TCA CTC
Gly Val Val Pro Gly Ile Pro Glu Pro Cys Cys Val Pro Glu Lys MET Ser Ser Leu

                1670                1685                1700
AGT ATT TTA TTC TTT GAT GAA AAT AAG AAT GTA GTG CTT AAA GTA TAC CCT AAC ATG
Ser Ile Leu Phe Phe Asp Glu Asn Lys Asn Val Val Leu Lys Val Tyr Pro Asn MET

  1715                1730    (472)     1746      1756      1766      1776
ACA GTA GAG TCT TGC GCT TGC AGA TAACCTGGCA AAGAACTCAT TTGAATGCTT AATTCAATCT
Thr Val Glu Ser Cys Ala Cys Arg

      1786
CTAGAGTCGA CGGAATTC
```

EXAMPLE VI

Expression of BMP-3

In order to produce bovine, human or other mammalian bone inductive factors, the DNA encoding it is transferred into an appropriate expression vector and introduced into mammalian cells or other preferred eukaryotic or prokaryotic hosts by conventional genetic engineering techniques. However the presently preferred expression system for biologically active recombinant human bone inductive factor is stably transformed mammalian cells.

One skilled in the art can construct mammalian expression vectors by employing the sequence of Tables I A and I B and II or other modified sequences and known vectors, such as pCD [Okayama et al., Mol. Cell Biol., 2:161-170 (1982)] and pJL3, pJL4 [Gough et al., EMBO J., 4:645-653 (1985)]. The transformation of these vectors into appropriate host cells can result in expression of a BMP-3 protein. One skilled in the art could manipulate the sequences of Tables I A and I B and II by eliminating or replacing the mammalian regulatory sequences flanking the coding sequence with bacterial sequences to create bacterial vectors for intracellular or extracellular expression by bacterial cells. For example, the coding sequences could be further manipulated (e.g. ligated to other known linkers or modified by deleting non-coding sequences therefrom or altering nucleotides therein by other known techniques). The modified BMP-3 coding sequence could then be inserted into a known bacterial vector using procedures such as described in T. Taniguchi et al., Proc. Natl Acad. Sci. USA, 77:5230-5233 (1980). This exemplary bacterial vector could then be transformed into bacterial host cells and BMP-3 expressed thereby. For a strategy for producing extracellular expression of BMP-3 in bacterial cells., see, e.g. European patent application EPA 177,343.

Similar manipulations can be performed for the construction of an insect vector [See, e.g. procedures described in published

European patent application 155,476] for expression in insect cells. A yeast vector could also be constructed employing yeast regulatory sequences for intracellular or extracellular expression of the factors of the present invention by yeast cells. [See, e.g., procedures described in published PCT application WO86/00639 and European patent application EPA 123,289].

A method for producing high levels of a BMP-3 protein factor of the invention from mammalian cells involves the construction of cells containing multiple copies of the heterologous BMP-3 gene. The heterologous gene can be linked to an amplifiable marker, e.g. the dihydrofolate reductase (DHFR) gene for which cells containing increased gene copies can be selected for propagation in increasing concentrations of methotrexate (MTX) according to the procedures of Kaufman and Sharp, J. Mol. Biol., 159:601-629 (1982). This approach can be employed with a number of different cell types. For example, a plasmid containing a DNA sequence for a BMP-3 protein of the invention in operative association with other plasmid sequences enabling expression thereof and the DHFR expression plasmid pAdA26SV(A)3 [Kaufman and Sharp, Mol. Cell. Biol., 2:1304 (1982)] can be co-introduced into DHFR-deficient CHO cells, DUKX-BII, by calcium phosphate coprecipitation and transfection, electroperation or protoplast fusion. DHFR expressing transformants are selected for growth in alpha media with dialyzed fetal calf serum, and subsequently selected for amplification by growth in increasing concentrations of MTX (sequential steps in 0.02, 0.2, 1.0 and 5uM MTX) as described in Kaufman et al., Mol Cell Biol., 5:1750 (1983). Transformants are cloned, and biologically active BMP-3 expression is monitored by rat bone formation assay described above in Example III. BMP-3 expression should increase with increasing levels of MTX resistance. Similar procedures can be followed to produce other BMP-3 family proteins.

A. COS Cell Expression

As one specific example of producing a BMP-3 protein of Example V, the insert of H128-4 is released from the vector arms by digestion with KpnI, blunting with T4 polymerase, ligating on an EcoR1 adapter, followed by digestion with Sal I. The insert is subcloned into the EcoR1 and Xho I cloning sites of the mammalian expression vector, pMT2CXM. Plasmid DNA from this subclone is transfected into COS cells by the DEAE-dextran procedure [Sompayrac and Danna PNAS 78:7575-7578 (1981); Luthman and Magnusson, Nucl.Acids Res. 11: 1295-1308 (1983)] and the cells are cultured. Serum-free 24 hr. conditioned medium is collected from the cells starting 40-70 hr. post-transfection.

The mammalian expression vector pMT2 CXM is a derivative of p91023 (b) (Wong et al., Science 228:810-815, 1985) differing from the latter in that it contains the ampicillin resistance gene in place of the tetracycline resistance gene and further contains a XhoI site for insertion of cDNA clones. The functional elements of pMT2 CXM have been described (Kaufman, R.J., 1985, Proc. Natl. Acad. Sci. USA 82:689-693) and include the adenovirus VA genes, the SV40 origin of replication including the 72 bp enhancer, the adenovirus major late promoter including a 5' splice site and the majority of the adenovirus tripartite leader sequence present on adenovirus late mRNAs, a 3' splice acceptor site, a DHFR insert, the SV40 early polyadenylation site (SV40), and pBR322 sequences needed for propagation in E. coli.

Plasmid pMT2 CXM is obtained by EcoRI digestion of pMT2-VWF, which has been deposited with the American Type Culture Collection (ATCC), Rockville, MD (USA) under accession number ATCC 67122. EcoRI digestion excises the cDNA insert present in pMT2-VWF, yielding pMT2 in linear form which can be ligated and used to transform E. coli HB 101 or DH-5 to ampicillin resistance. Plasmid pMT2 DNA can be prepared by conventional

methods. pMT2CXM is then constructed using loopout/in mutagenesis (Morinaga,et al., Biotechnology 84: 636 (1984). This removes bases 1075 to 1145 relative to the starting from thr Hind III site near the SV40 origin of replication and enhancer sequences of pMT2. In addition it inserts the following sequence:

5' PO_CATGGGCAGCTCGAG-3'

at nucleotide 1145. This sequence contains the recognition site for the restriction endonuclease Xho I, which is compatable with the Sal I site on the BMP-3 insert. Plasmid pMT2 CXM DNA may be prepared by conventional methods.

B. CHO Cell Expression

A BMP-3 protein of Example V may be expressed in CHO cells by releasing the insert of H128-4 from the vector arms by digestion with KpnI, blunting with T4 polymerase, ligating on an EcoR1 adapter, followed by digestion with Sal I. The insert is subcloned into the EcoR1 and Xho I cloning sites of the mammalian expression vector, pMT2CXM. Plasmid DNA from this subclone is transfected into CHO cells by electroporation [Neuman et al, EMBO J., 1:841-845 (1982)]. Two days later, cells are switched to selective medium containing 10% dialyzed fetal bovine serum and lacking nucleosides. Colonies expressing DHFR are counted 10-14 days later. Individual colonies or pools of colonies are expanded and analyzed for expression of BMP-3 RNA and protein using standard procedures and are subsequently selected for amplification by growth in increasing concentrations of MTX.

cDNA genes inserted into the EcoRI and/or Xho I sites will be expressed as a bicistronic mRNA with DHFR in the second position. In this configuration, translation of the upstream (BMP-3) open reading frame is more efficient than the downstream (DHFR) cDNA gene [Kaufman et al, EMBO J. 6:187-193 (1987). The amount of DHFR protein expressed is nevertheless sufficient for selection of stable CHO cell lines.

Characterization of the BMP-3 polypeptides through pulse labeling with [35S] methionine and polyacrylamide gel electrophoresis indicates that multiple molecular size forms of BMP-3 proteins are being expressed and secreted from the stable CHO lines.

Example VII

Biological Activity of Expressed BMP-3

To measure the biological activity of the expressed BMP-3 obtained in Example VI above, the BMP-3 is partially purified on a Heparin Sepharose column. 4 ml of the collected post transfection conditioned medium supernatant from one 100 mm dish is concentrated approximately 10 fold by ultrafiltration on a YM 10 membrane and then dialyzed against 20mM Tris, 0.15 M NaCl, pH 7.4 (starting buffer). This material is then applied to a 1.1 ml Heparin Sepharose column in starting buffer. Unbound proteins are removed by an 8 ml wash of starting buffer, and bound proteins, including BMP-3, are desorbed by a 3-4 ml wash of 20 mM Tris, 2.0 M NaCl, pH 7.4.

The proteins bound by the Heparin column are concentrated approximately 10-fold on a Centricon 10 and the salt reduced by diafiltration with 0.1% trifluoroacetic acid. The appropriate amount of this solution is mixed with 20 mg of rat matrix and then assayed for in vivo bone and/or cartilage formation activity by the Rosen-modified Sampath - Reddi assay. A mock transfection supernatant fractionation is used as a control for COS expressed proteins and for CHO expressed proteins CHO cell without BMP-3 conditioned medium fractionation is utilized. The implants containing rat matrix to which specific amounts of human BMP-3 have been added are removed from rats after seven days and processed for histological evaluation. Representative sections from each implant are stained for the presence of new bone mineral with von Kossa and acid fuschin, and for the presence of cartilage-specific matrix formation using toluidine blue.

The types of cells present within the section, as well as the extent to which these cells display phenotype are evaluated and scored as described in Example III.

Addition of human BMP-3 to the matrix material resulted in formation of cartilage-like nodules at 5 days post implantation. The chondroblast-type cells were recognizable by shape and expression of metachromatic matrix. The assay results indicate that BMP-3 proteins may be characterized by the ability of 1μg of the protein to score at least +2 in the rat bone formation assay. The amount of activity observed for human BMP-3 indicates that it may be dependent upon the amount of BMP-3 protein added to the matrix sample.

The procedures described above may be employed to isolate other related BMP-3 factors of interest by utilizing the bovine BMP-3 or human BMP-3 factors as a probe source. Such other BMP-3 proteins may find similar utility in, inter alia, fracture repair, wound healing and tissue repair.

The foregoing descriptions detail presently preferred embodiments of the present invention. Numerous modifications and variations in practice thereof are expected to occur to those skilled in the art upon consideration of these descriptions. Those modifications and variations are believed to be encompassed within the claims appended hereto.

What is claimed is:

1. A purified BMP-3 protein produced by the steps of
(a) culturing a cell transformed with a cDNA substantially as shown in Table II; and
(b) recovering from said culture medium a protein containing substantially the 96 amino acid sequence as shown in Table II from amino acid # 377 to amino acid # 472.

2. A protein of claim 1 further characterized by the ability to induce cartilage and/or bone formation.

3. A protein of claim 1 further characterized by the ability of 1μg of said protein to score at least C +2 in the Rosen-modified Sampath-Reddi assay.

4. A cDNA sequence encoding a protein of claim 2.

5. A host cell transformed with a cDNA of claim 4.

6. A method for producing a purified BMP-3 protein said method comprising the steps of
(a) culturing in a suitable culture medium said transformed host cells of claim 5; and
(b) isolating and purifying said BMP-3 from said culture medium.

7. A pharmaceutical composition comprising an effective amount of a protein of claim 1 in admixture with a pharmaceutically acceptable vehicle.

8. A pharmaceutical formulation for bone and/or cartilage formation comprising an effective amount of a protein of claim 2 in a pharmaceutically acceptable vehicle.

9. A composition of claim 8 further comprising a matrix for supporting said composition and providing a surface for bone and/or cartilage growth.

10. The composition of claim 9 wherein said matrix comprises a material selected from the group consisting of hydroxyapatite, collagen, polylactic acid and tricalcium phosphate.

11. A method for inducing bone and/or cartilage formation in a patient in need of same comprising administering to said patient an efective amount of the composition of claim 8.

12. A pharmaceutical composition for wound healing and tissue repair said composition comprising an effective amount of the protein of claim 1 in a pharmaceutically acceptable vehicle.

13. A method for treating wounds and/or tissue repair in a patient in need of same comprising administering to said patient an effective amount of the composition of claim 12.

14. An isolated DNA sequence encoding a BMP-3 protein said DNA sequence comprising substantially the nucleotide sequence or a portion thereof selected from the group consisting of:

(a) nucleotide #321 through nucleotide #1736

(b) sequences which

(1) hybridize to said sequence under stringent hybridization conditions; and

(2) encode a protein characterized by the ability to induce cartilage and/or bone formation.

15. A DNA sequence of claim 14 further characterized by the ability of 1μg of said protein having the ability to score at least +2 in the Rosen-modified Sampath-Reddi assay.

16. A vector comprising a DNA sequence of claim 14 in operative association with an expression control sequence therefor.

17. A host cell transformed with a DNA sequence of claim 14.

18. A method for producing a BMP-3 protein, said method comprising the steps of

(a) culturing in a suitable culture medium said transformed host cell of claim 17; and

(b) isolating and purifying said BMP-3 from said culture medium.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 89/01464

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12P 21/00; C07K 13/00; C07H 15/12

US: 435/68; 530/350; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US	435/68,91,172.1,172.3,320 536/27;530/350;935/18,22,33,56

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Chemical Abstracts Data Base (CAS) 1967-1989

Key Words: bone morphogenic protein, pharmaceutical composition

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US,A, 4,619,989 (URIST)28 October 1986, see abstract.	1-18
P,Y	US,A, 4,795,804 (URIST)03 January 1989, see abstract.	1-18
Y,P	Proceedings of the National Academy of Sciences, U.S.A., Volume 85, issued December 1988 (WANG et al) "Purification and characterization of other distinct bone-inducing factors", see pages 9484-9488, see especially, the absract.	1-18
Y	WO,A W086/00525 (SZABO)30 January 1986 see abstract.	12-13
Y	US,A, 4,394,370 (JEFFRIES) 19 July 1983 see abstract.	7-13

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
09 June 1989	17 JUL 1989
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Joan Ellis

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US,A, 4,563,350 (NATHAN et al)07 January 1986, see abstract.	7-13
Y,P	US,A, 4,789,732 (URIST)06 December 1988, see abstract.	7-13